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TITLE: Breast Cancer and Early Onset Childhood Obesity: cell Specific Gene Expression in Mammary Epithelia and Adipocytes

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14. ABSTRACT

Obesity has become a major health problem in children and adults and is associated with increased breast cancer incidence and mortality. The epidemic of childhood obesity is recent and little information exists regarding its association with mammary tumorigenesis. Towards better understanding this relationship we have developed and characterized a new rat model of childhood onset Diet Induced Obesity (DIO) and breast cancer. We have shown that young female rats fed a high fat Western Style diet have a 2-fold higher body fat mass and elevated serum comorbidity factors as compared to Chow fed Lean rats. When these animals are treated with the carcinogen MNU, mammary tumors appear sooner and in greater numbers in Obese rats. We determined via histology that tumors from Obese rats are of a more invasive type compared to tumors from Lean rats. This is in accord with the association between human obesity and breast cancer mortality. This new model parallels the onset of obesity as it occurs in humans and therefore provides an excellent system to study the underlying mechanisms of obesity and mammary tumor formation and progression. Our long-term goals are to exploit this model to better understand adipocyte-epithelial interactions during mammary tumorigenesis, identify and validate novel molecular therapeutic targets, and to establish biomarkers for cancer prevention and prognosis.

15. SUBJECT TERMS
Obesity, Breast Cancer, Western Diet, rat, MNU

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Introduction

Obesity is a major health problem around the world and is positively associated with breast cancer incidence and mortality (1). Recent rapid increases in childhood obesity indicate the adverse effects of obesity will be a concern for decades to come (2). To better understand the relationship between childhood obesity and breast cancer, we have developed and characterized a new rat model of early onset Diet Induced Obesity (DIO). In this model rats are fed a Western Diet that is high in fat and higher in simple carbohydrate compared to diets of traditional DIO studies. This more closely represents the typical human diet of North America. We have shown that young female rats fed a Western diet have a higher body fat mass and elevated serum comorbidity factors as compared to Chow fed Lean rats. Furthermore, MNU-induced mammary tumors appear sooner, in greater numbers and are more invasive in Obese rats as compared to tumors from Lean rats. This is in accord with the association between human obesity and breast cancer mortality. This new model provides an excellent system to identify the mechanisms of obesity towards mammary tumorigenesis, to better understand adipocyte-epithelial interactions and to establish biomarkers for cancer prevention and prognosis.

Body

Development of a Novel Rat model of Diet Induced Obesity (DIO)

Rational for a New model of Female Obesity

In humans obesity is associated with increased breast cancer incidence and morbidity (1). To better understand the mechanisms involved in obesity-associated breast cancer, it is essential to establish a model of obesity that parallels the human condition. In our recently developed model, out bred female Sprauge-Dawley rats are fed a "Western Diet" beginning at weaning. This model was chosen for several reasons. First, the Western diet used is high in fat and higher in simple carbohydrates as compared to traditional high fat diets used in animal studies and more closely represents a typical human diet of North America and Europe (Table 1). Second, the most widely used rodent models of obesity are genetically altered and devoid of the obesity hormone leptin (*ob/ob* mice) or its receptor (*db/db* mice, Zucker rat). These leptin signaling impaired animals are resistant to oncogene and chemically induced mammary tumors (3,4). In general, human obesity is not caused by mutations in leptin or its receptor (5). As expression of leptin and its receptor remain intact in our rat model, it more closely represents human obesity. Third, in this model obesity is determined by Dual Xray Absorbimetry (DEXA) scan and not by body weight. This method allows the measurement of body composition of animals, in particular % body fat mass. Given that two animals having the same weight can differ vastly in their adiposity, DEXA scan provides a substantial advantage compared to scale weight measurements. Finally, the model we have developed mimics childhood obesity in humans, a trend that has dramatically increased in recent years (2). Compared to lean children, children who are obese are far more likely to become obese adults. This disturbing trend is an indicator that obesity-related cancers will be a concern for decades to come.

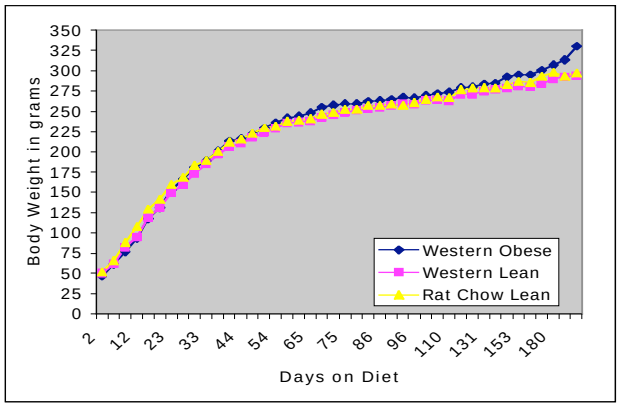
Proportion of rats susceptible to DIO

Current data, based on BMI, reveals that 30% of the American population is considered clinically obese (6). Given their similarity in physiology and genetics to humans, it is likely that within a population of outbred rats, a proportion of rats would be susceptible to DIO. Indeed this was shown to be true by Ghibaudi *et al.* (7) where outbred Sprague-Dawley male rats were fed a Western diet beginning at 50 days old. After 6 weeks, approximately 15-30% of the Western diet fed rats gained a significantly higher amount of weight compared to control fed animals. Based on this study, we tested if this phenomenon could occur in female Sprague-Dawley rats. Establishing that the Western diet can consistently induce obesity in a subpopulation of outbred female Sprague-Dawley rats was essential towards establishing our *in vivo* model of obesity-associated breast cancer. In our preliminary study, female Sprague-Dawley rats weaned at 21 days old were randomly placed on either a standard Rat Chow Diet (LabDiet) or a Western Diet (Diet Research), shown in Table 1.

	Western	Medium Fat	High Fat
Protein	**17**	**17**	**20**
Casein	16.6	16.5	19.7
Carbohydrate	**43**	**51**	**35**
Corn starch	4.3	18.7	7.2
Sucrose	29.1	25.2	17
Maltodextrin	8.5	6.5	10
Fat	**41**	**32**	**45**
Milk fat	38.4	0	0
Corn oil	2	23.1	0
Soybean oil	0	0	5.5
Lard	0	0	39.5
Butter fat	0	8.7	0

Table 1. Major composition of commonly used DIO Diets by calories in percent. Comparison of Western Diet with representative medium and high fat diets (Ghibaudi, et al., Obesity Research, pp 956-963 10(9):2002). Total percent kilocalories as protein, carbohydrate and fat are broken down by dietary composition. Kcal/g Western Diet = 4.7, Medium Fat Diet = 4.41, and High Fat Diet = 4.73.

All rats were weighed 2 times a week and their food intake was monitored until 54 days of age. Animals were then DEXA scanned (Dual Xray absorbimetry) to determine body fat mass composition. As shown in Table A, rats were ranked and then grouped into three categories based on % body fat mass, Obese W = Obese rats on western diet: Lean W = Lean rats on western diet: Lean RC = Lean rats on rat chow diet served as the Control group. In our DEXA scan data (Table 2 A) we show that the Western diet used induces a statistically significant increase in body fat mass as compared to the Rat Chow diet. Approximately 30% of the Western Diet fed rats had a 2-fold increase in % body fat mass as compared to Chow fed rats. Interestingly, approximately 30% of the Western fed rats had the same % fat mass (designated Lean Western) as the Rat Chow fed animals. In an identical but separate study we weighed, monitored food intake and DEXA scanned Western and Chow fed rats after 25 weeks on respective diets. Results from this study demonstrate that a 2-fold difference in body fat mass between Obese Western and Lean Rat Chow animals is maintained through 6.5 months (Table 2 B). As seen with the shorter study, a subset of the Western fed animals had a statistically similar % fat mass as the Rat Chow rats. Figure 1 indicates that after 25 weeks on their respective diets, Obese Western rats have a higher body weight as compared to the Lean Rat Chow Group. Figure 2 demonstrates that Rat Chow fed animals consume more food in grams, compared to the Western fed group. However, when food intake is expressed as Kcal, we show that rats in each group consume the same amount of calories per day. This suggests that the obesity induced in the Obese rats in not the result of behavioral differences such as hyperphagia. To further characterize our model, we also measured several circulating co-morbidity factors associated with human obesity in the three groups of rats, including Leptin, Free fatty acids (FFA), triglycerides (TG) and insulin. Figure 3 shows that Obese Western rats had 2-3 fold higher serum leptin and higher TG, FFA and insulin levels as compared to Lean RC animals. The information derived from these preliminary studies was essential in planning the subsequent mammary tumorigenesis experiments. We determined the number of Obese rats that could be produced using the Western diet. We also demonstrated that we could see significant differences in % fat mass between groups at 54 days, the optimal age of effectiveness for inducing mammary tumors with MNU.



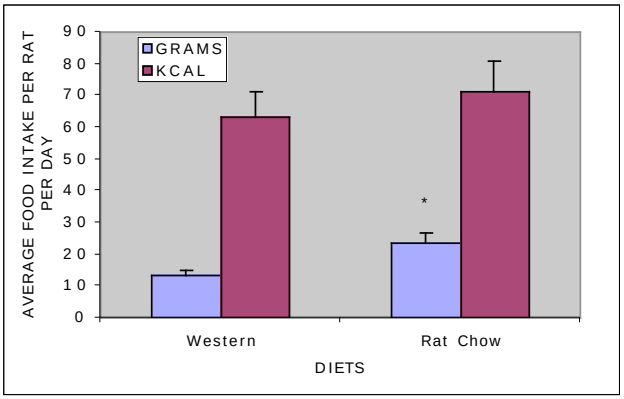


Figure 1. Body weight gain in Western Obese, Western Lean and Rat Chow Lean groups. Body weight was measured bi weekly for 6 1/2 months. At this time rats were DEXA scanned and placed into respective groups based on % body fat mass. Data shown is mean ± sem from day 1 of Western or Rat Chow Diet regimens. n= 12-15 for each group.

Figure 2. Average Food and energy Intake of female SD rats on Western and Rat Chow diets. Food intake as total grams per rat per day and as Kcal/g metabolizable energy intake per rat per day over 6 1/2 months. Data represents Mean ± sem, in Rats on Western Diet consumed significantly less food intake (in grams) as compared to Rat Chow Diet fed animals, *p ≤ 0.05.

A

Diet	Scale BW(g)	DXA BW(g)	Bone Mass(g)	Fat Mass (%)	Lean Mass (%)
Obese W	185.4 ± 5.6	200.8 ± 5.3	2.5 ± 0.2	**8.6 ± 1.0**	**91.4 ± 1.0**
Lean W	171.7 ± 7.3	179.3 ± 7.2	2.7 ± 0.2	4.2 ± 0.1*	95.8 ± 0.1*
Lean RC	162.8 ± 4.0	176.4 ± 4.3	2.8 ± 0.2	4.0 ± 0.1*	96.0 ± 0.0*

B

Diet	Scale BW(g)	DEXA BW(g)	Bone Mass(g)	Fat Mass (%)	Lean Mass (%)
Obese W	320.3 ± 9.0	338.8 ± 5.9	8.5 ± 0.4	**17.4 ± 1.1***	**82.6 ± 1.1***
Lean W	293.6 ± 4.2	315.2 ± 3.7	8.0 ± 0.3	9.2 ± 1.2	90.8 ± 1.2
Lean RC	297.1 ± 2.9	316.4 ± 4.0	8.5 ± 1.6	9.0 ± 1.6	91.0 ± 1.6

Table 2. Body Mass Composition in Western and Chow Fed Rats. The above table summarizes the body mass composition of our three categories of rats after 54 days (A) and 25 weeks (B) of diet regimens. Obese W = Obese rats on western diet: Lean W = Lean rats on western diet: Lean RC = Lean rats on rat chow diet; BW = body weight. The first and second columns represent weight of rats as determined by triple beam scale and Dual Xray absorbimetry (DEXA), respectively. Bone mass is represented in grams whereas fat mass and lean mass are expressed as percents. Values are mean ± SEM n = 12-15 per group * $p < 0.05$ between obese and lean groups.

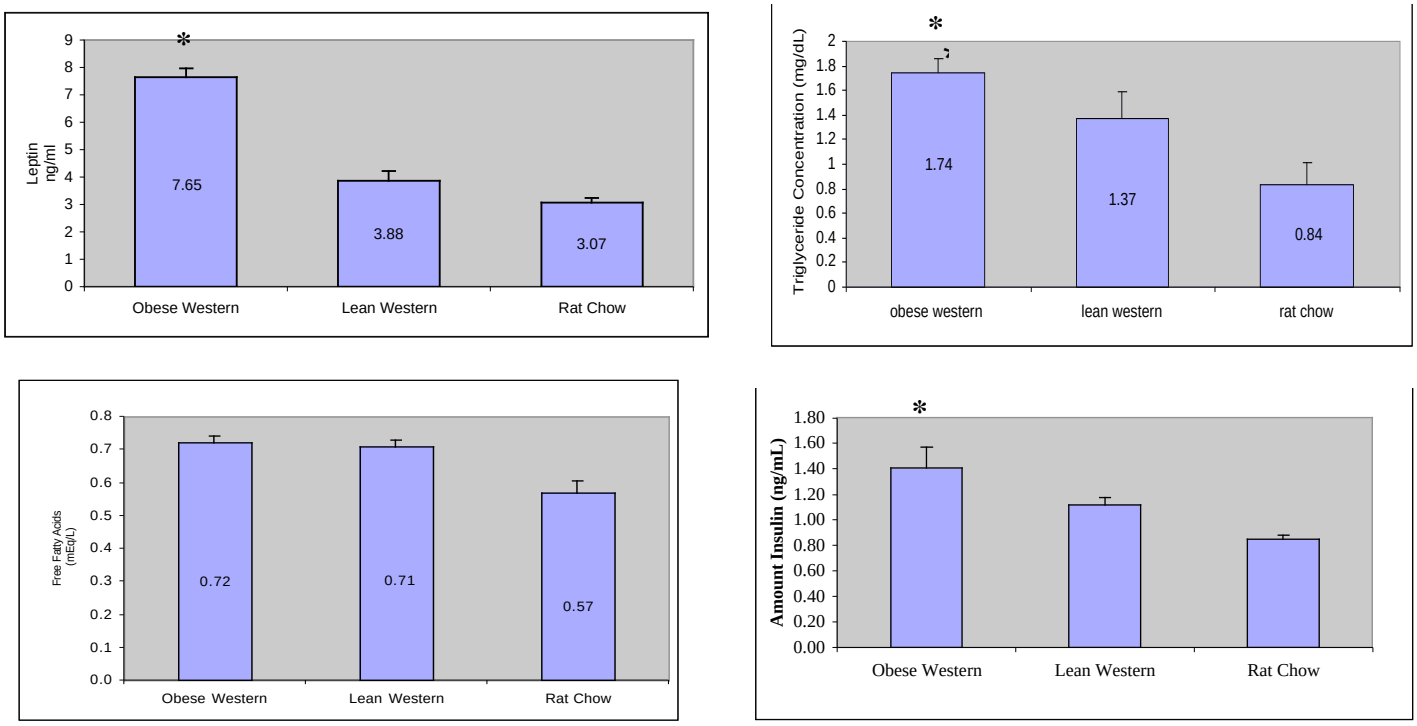


Figure 3. Serum Comorbidity factors in Obese and Lean Western diet and Lean Rat Chow diet rats. Female rats were weaned at 21 days old and randomly placed on either a Western Diet (Diet Research) or a Rat Chow diet (LabDiet). At 25 weeks DEXA Scans were performed to determine body fat composition and define all three groups. Rats with the highest body fat mass were assigned to the Obese Western (DIO) group, (n=13). Rats with the lowest fat mass were designated Lean Western (n=12). Rat Chow fed animals had an n=15. Immediately after scanning rats were sacrificed and blood serum was isolated. Serum Leptin and Insulin levels were measured via the rat RIA kit (Linco). Serum free fatty acids and triglycerides were measured via colorimetric assay (Sigma). Data represents means $\pm$ SE. * = p 0.05 or less.

Onset of puberty in DIO rats

In humans it is well established that the onset of puberty is dependent in part on the accumulation of body fat. A classic example is the delayed menarche seen in lean young women that are long distance runners (8). Over the past century the average age at menarche of American women has decreased from 14.2 yrs to 12.5 yrs (9). This secular trend has been attributed to improved environmental conditions, improved nutrition and obesity. With these evidences in mind, we hypothesized that in our rat DIO model the Western Diet fed animals would undergo puberty at an earlier age compared to Rat Chow fed Controls. We tested this hypothesis by evaluating vaginal introitus (vaginal opening), a commonly used indicator of estrus cycle onset in the rodents described above (10). In Figure 4 we show that at 43 days of age, 100% of the Western Diet fed rats and only 60% of the Rat Chow fed animals showed vaginal opening. Not until day 53 did 100% of Rat Chow fed controls exhibit vaginal opening. These results indicate that, compared to Rat Chow fed controls, our Western diet induces an earlier onset of puberty in young female Sprague-Dawley rats. This model follows the same trend in humans where childhood obesity leads to an earlier age of menarche in females.

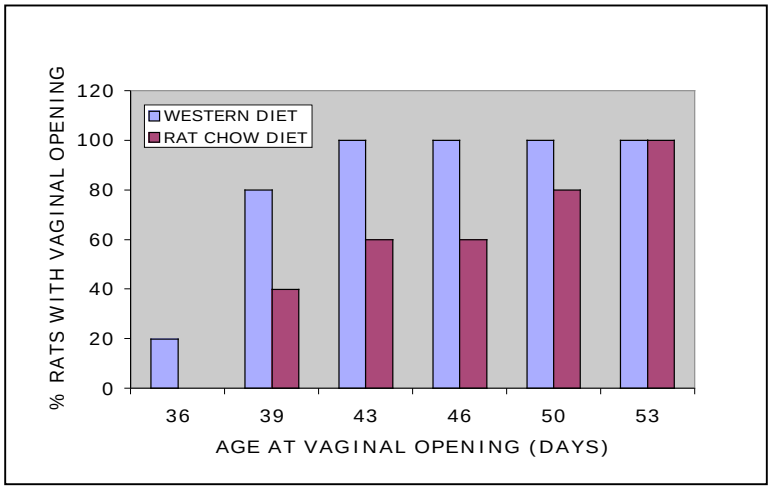


Figure 4. Age of puberty onset in Western diet fed female rats. Onset of puberty was determined by vaginal opening (VO). Female rats were weaned at 21 days old and randomly placed on either a Western Diet (Diet Research) n=15 or Rat Chow Diet (LabDiet) n=10. Western fed rats were ranked according to weight. Graph represents the % of total rats with VO on Western diet, and on Rat Chow diet.

Mammary Tumor incidence in Western diet fed rats

In a recent study we have evaluated the onset of MNU induced mammary tumors in our rat model of DIO. As described above we produced Obese Western (n=13), Lean Western (n=13) and Lean Rat Chow (n=12) SD female rats and at 65 days old each group was injected with Methylnitrosourea (MNU). The development of mammary tumors via MNU treatment is a widely accepted model of carcinogenesis (11). Recent molecular profiling studies have revealed that MNU-induced rat tumors are similar to low-grade human mammary tumors and are an excellent model for breast cancer (12). Another major advantage of this system is that hormone dependent tumors are produced which can progress to the hormone independent state, as is common in human breast cancer (11). After injection the three groups of rats were palpated twice a week and tumor load and latency period were recorded. When comparing the three groups, significant differences in tumor onset were observed after 25 weeks post MNU injection. Briefly, mammary tumors appeared sooner and in greater numbers in Obese and Lean Western fed rats as compared to Chow fed Lean rats (Figure 5 and Table 3). Histological analysis of tumors revealed that collectively the Obese group had the highest percentage of hi-grade mammary carcinoma tumors (92% of total tumor number) as compared to the other two groups (50%) (Table 3 and Figure 6). These data demonstrate that mammary tumorigenesis is more aggressive in Obese compared to Lean rats. Also we delineate that consumption of a Western diet, in the absence of obesity, may lead to a sooner onset of mammary tumor formation.

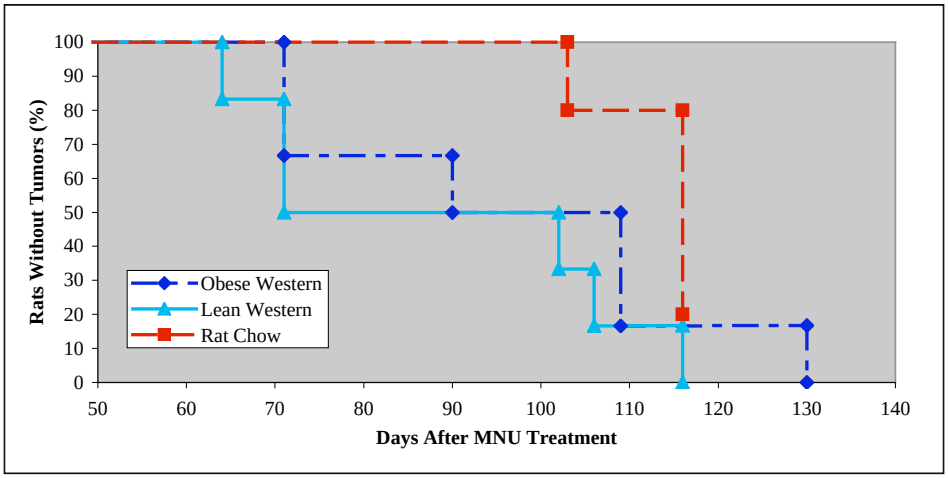


Figure 5. Influence of Obesity and Western Diet on mammary tumorigenesis in Sprague-Dawley Rats. Rats were placed on Western or Rat Chow Diets as described previously. Based on % body fat mass (DEXA scan at 54 days) rats were placed into three groups; Western Diet Obese (n=13), Lean Western (n=13) and Lean Rat Chow controls (n=12). At 64 days of age animals in each group were anesthetized and given a single intraparitoneal injection of MNU (50mg MNU/kg body weight). All rats were palpated twice a week to detect mammary tumors. Mammary tumor load and latency period are presented in graph and table form below.

Table 3. Mammary tumor development in Sprague Dawley rats 25wks after MNU injection

Diet	Group	# Rats	Detection of First Tumor (Day)	Total Number of Tumors	Histology (# of Hi grade carcinomas: # of adenomas*)	Hi grade Carcinomas (% per total # of tumors)
Western	Obese	7	71	24	22:2	92%
Western	Lean	7	74	18	9:6	50%
Rat Chow	Lean	7	109	14	7:3	50%

*Remainder of tumors in each group were Lo grade carcinomas.

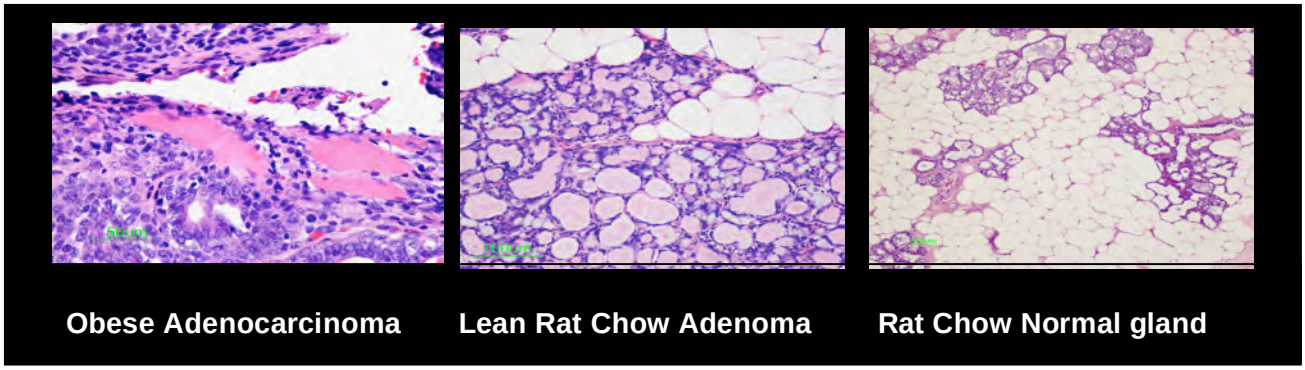


Figure 6. Influence of Obesity and Western Diet on mammary tumorigenesis in Sprague-Dawley Rats. As described above three groups of rats were produced; Western Diet Obese, Lean Western and Lean Rat Chow controls. Animals in each group were given a single injection of MNU (50mg MNU/kg body weight). At 25 wks tumors and mammary tissue were collected and prepared for histological analysis via hematoxylin and eosin staining. Predominantly, more aggressive mammary tumors appeared in the Obese group. The left panel shows an adenocarcinoma from an Obese rat where tumor cells (purple) are invading adjacent muscle tissue (pink). In the middle panel a benign Adenoma, with adjacent fat cells (white), from a Lean rat is shown. On the right is a tumor-free gland from a lean rat. This gland consists primarily of fat cells with a few primary epithelial ducts (purple).

Key Research Accomplishments

Model of Childhood Obesity

- We have developed and characterized a new female rat model of Childhood onset Diet Induced Obesity (DIO).
- Using outbred Sprague Dawley rats we have shown that, compared to Rat Chow, consumption of a Western diet caused increased body fat mass in approximately 20-30% of animals within 54 days. Obese rats had a 2-fold higher % fat mass as compared to Chow Fed rats.
- We demonstrate that the 2-fold difference in % fat mass between the Obese Western and Lean Rat Chow animals was maintained through 25 weeks.
- We showed that animals on Rat Chow consumed more food in *grams* per day, as compared to Western Diet fed rats. However, Rat Chow and Western fed groups had the same caloric intake per day over 25 weeks.
- Vaginal opening, and indicator of puberty onset, occurred earlier in the Western Diet group as compared to Rat Chow fed animals.
- As in human obesity, we showed that Obese Western rats had 2-3 fold higher serum leptin and higher TG, FFA and insulin levels, compared to Lean Rat Chow animals.

Key Research Accomplishments, Continued

Mammary Tumorigenesis in Early Onset Obese Rats

- We demonstrate that in Western Diet fed rats, MNU-induced mammary tumors appear sooner as compared to Rat Chow animals.
- We have shown that Obese Western rats had greater numbers of mammary tumors than Lean Western and Lean Rat Chow animals.
- Based on histological classification, we demonstrate that Obese Western animals have predominately hi-grade invasive type mammary tumors compared to Lean rats on the Western or Rat Chow diets.

Unexpected delays in proposed work/remaining Task

The above accomplishments represent the majority of Task 1 as outlined in our grant proposal. We did experience unforeseen delays in our work, as there was problems with DEXA scan equipment malfunctions. Furthermore, there were restrictions regarding the number of animals that could be scanned within a given number of days. For the latter reason we were forced to break up our study into two repeats with less animals per repeat (instead of performing our entire study at all at once). Currently, we have completed all the necessary animal studies and have collected all necessary tissues to complete our project. To complete Task 1 we are currently analyzing serum comorbidity factors in tumor-free and tumor-bearing rats. We are also analyzing mammary tissues and tumors for estrogen receptor content via immunohistochemistry. Simultaneously, towards Task 2 we preparing mammary tissues and tumors for laser capture microdissection. This will be followed by epithelial and adipocyte cell specific RNA isolation and Affymetrix array gene chip analyses (as described in our proposal).

Reportable Outcomes

Presentations and Abstracts

I.G. Camarillo, C. Rehrer, Chris Gottfried and M. Nichols. "Mammary Tumorigenesis in a Novel Rat Model of Childhood Onset Diet-Induced Obesity"" Endocrine Society 88th Annual Conference, Boston MA, 2006. - *Abstract selected for Endocrine Society Conference Research Summaries Book; A listing of 100 newsworthy abstracts- over 3,000 abstracts submitted. Professor Camarillo also received a FASEB MARC Travel award to present this research.*

C. Rehrer, Chris Gottfried, M. Nichols and I.G. Camarillo. "A Female Rat Model of Childhood Onset Diet Induced Obesity" Peachy Breast Cancer Conference, Indiana University-Purdue University, Indianapolis, Indiana, February 9, 2006.

"The influence of Childhood Onset Obesity on Breast Cancer; A new animal model" Purdue University Oncological Sciences Center, Progress and Problems in Cancer Prevention and Control symposium. Purdue University, W. Lafayette, January 17, 2006.

Awards/Recognitions as a result of this grant

Endocrine Society Conference Research Summaries Book (newsworthy abstracts) 2006
FASEB MARC Travel Award, 2006
AACR Minority Scholar Award, 2006
Charles Rehrer, National Science Foundation Graduate Research Fellowship
Charles Rehrer**,** Graduate Student Fellowship Incentive Award
Hwei-Gene Chin (undergrad), Purdue/Howard Hughes Medical Institute Summer Internship
Julie Wilmowski (undergrad), Purdue/Howard Hughes Medical Institute Summer Internship

Reportable Outcomes, Continued

Funding applied for based on work supported by this grant

Agency / Title of Grant: NIH: KO1: Mechanisms of obesity associated mammary tumorigenesis

Duration of Funding: Five (5) years (2006-2011)

Total amount of award: $792,054 Role: PI

Agency / Title of Grant: NIH:R21 Tumor Inhibition by Adiponectin in New Models of Obesity-associated Breast Cancer

Duration of Funding: Two (2) years (2006-2008)

Total amount of award: $417,999 Role: PI

Agency / Title of Grant: NIH:RO3 Multiphoton Imaging to Evaluate the Influence of Dietary Fatty Acids on Mammary Tumorigenesis in Obese rats

Duration of Funding: Two (2) years (2007-2009)

Total amount of award: $152,500 Role: PI

Agency / Title of Grant: Purdue University Oncological Sciences Center; Multiphoton Imaging to Evaluate the Influence of Dietary Fatty Acids on Mammary Tumorigenesis in Obese rats

Duration of Funding: One (1) year (2006-2007)

Total amount of award: $50,000 Role: PI

Agency / Title of Grant: Geyer Foundation: Mechanisms of Obesity associated Breast Cancer

Duration of Funding: One (1) year (2006-2007)

Total amount of award: $100,000 Role: PI

Agency / Title of Grant: Purdue Research Foundation: Obesity and Breast Cancer

Duration of Funding: One (1) year (2006-2007)

Total amount of award: $50,000 Role: PI

Agency / Title of Grant: Komen Foundation: Validation of SHIP2 as a molecular target in obesity-linked cancer

Duration of Funding: Two (2) year (2006-2008)

Total amount of award: $250,000 Role: Co-PI

Conclusion

Obesity is a major health problem in the U.S. and is associated with increased breast cancer incidence and

mortality. The epidemic of childhood obesity is recent and little information exists regarding its association with breast cancer. Towards better understanding this relationship we have developed and characterized a new rat model of childhood onset Diet Induced Obesity (DIO) and breast cancer. We have shown that 20-30% of young female ourtbred rats on a Western diet have higher body fat mass and disregulated serum comorbidity factors, as compared to Chow fed Lean rats. This obese rat model better parallels the onset of obesity as it occurs in humans. We have shown that in Western Diet fed animals (Obese *and* Lean), MNU-induced mammary tumors appear sooner as compared to Rat Chow fed Lean rats. Furthermore, Obese rats had higher numbers of tumors than Chow fed rats. Via histology we determined that mammary tumors from Obese rats are predominately of an invasive phenotype, compared to tumors from Lean rats. This aggressive phenotype is in accord with the association between human obesity and breast cancer mortality. Interestingly, this work provides new information suggesting that consumption of a western diet by lean individuals may affect breast cancer risk. We also provide evidence that a Western diet in the context of obesity can influence tumor load and phenotype. As a scientific product, this new model provides an excellent system to study the underlying mechanisms of childhood obesity and mammary tumor formation and progression. Future studies will exploit this model to better understand adipocyte-epithelial interactions during mammary tumorigenesis, identify and validate novel molecular therapeutic targets, and to establish biomarkers for cancer prevention and prognosis.

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